

The effects of salt preconditioning on physiological, biochemical, and molecular responses of *Casuarina glauca* seedlings grown under salt stress

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Abstract

Salinity is one of the most harmful abiotic stresses with negative impacts on crop growth and development. Monitoring land salinization is, therefore, a major global issue. One of the suggested solutions to support rehabilitation programs is the use of fast-growing salt-tolerant woody species, like the tropical trees belonging to the *Casuarinaceae* family. Although salt preconditioning is a simple and practical method, its role in improving plant tolerance to salinity has still been scarcely applied. Thus, this study investigated the effect of salt preconditioning on different physiological, biochemical, and molecular mechanisms in *Casuarina glauca* seedlings grown in saline conditions. Preconditioned (PrS) and non-preconditioned (NPrS) seedlings were subjected to salt stress for 12 months. Gas exchange, electrolyte leakage, chlorophyll content, chlorophyll fluorescence, water relations parameters derived from pressure–volume curves, primary and secondary metabolism, and gene expression were assessed at the end of the experiment. Preconditioned plants promoted stronger tolerance to salt stress and improved gas exchange parameters, intrinsic water-use efficiency, and chlorophyll content compared to non-preconditioned plants. Also, membrane integrity was maintained in preconditioned plants but increased significantly in the absence of preconditioning. Salinity had no significant effect on the photochemical efficiency of PSII and resulted in a slight decrease in the initial fluorescence (F_o) in either treatment when compared to control. The results indicate an increase in secondary metabolism as shown by the enhancement of phenolic content as well as an increase in the expression of salt tolerance-related genes (*GPX1*, *SOD1*, *SHD*, *APX*, and *GAPHD*). Our findings also showed a considerable accumulation of soluble sugar and proline under salt stress, with a higher accumulation in preconditioned plants, confirming their salt tolerance and performance under saline conditions. Overall, these results indicate that *C. glauca* is highly suitable to be used on salinity-affected soils and can develop a higher tolerance to salt stress after preconditioning treatments.

Introduction

Worldwide, agricultural productivity is severely hampered by many environmental pressures such as global warming, salinity, and drought (Tester and Langridge, 2010; Rizwan et al., 2015). Among these abiotic factors, soil salinity is one of the most devastating environmental stresses, causing major declines in cultivated land area, crop productivity and quality of crops. For instance, saline soils are estimated to cover 16 million hectares in the Mediterranean region (Munns, 2002) and 1.5 million hectares in Tunisia, accounting for nearly 10% of the country's total area (Hachicha, 2007). Secondary salinization due to anthropogenetic activities further increases the problem of salinity. It has been estimated that 20% of total worldwide cultivated land and 33% of irrigated agricultural lands are already affected by high salinity, causing around US\$27.3 billion per year in economic losses (Qadir et al., 2004). Soil salinization has a high environmental, ecological, economic, and social impact, shrinking agricultural lands and causing losses in agricultural productivity (Kumar and Sharma, 2020). Salinity stress can cause a variety of physiological problems, including osmotic effects, ion-specific stress, ionic imbalance, and oxidative stress (Tabatabaei and Ehsanzadeh, 2016), which may result in membrane lipoperoxidation and membrane injury (Leshem, 1987; Zhu, 2001). Osmotic stress leads to inhibition of water uptake, which in turn inhibits cell expansion and cell wall biosynthesis, stomatal conductance, protein synthesis, photosynthetic activity, and lateral bud

development (Munns and Tester, 2008). Plants deal with salt stress in a variety of ways that include a mix of stress avoidance and tolerance mechanisms (Dias et al., 2010). However, salt-tolerant species are able to induce molecular and cellular changes that counteract the effect of osmotic stress. These operate at multiple levels. Many genes related to salt-stress responses are differentially expressed after plants detect salt-stress signals (Zhu, 2001) to help improve plant functionality while avoiding extensive negative effects (Dias et al., 2010). Also, under salinity, many changes occur at physiological and biochemical levels (Guo et al., 2012; Kong et al., 2016), including stomatal closure, accumulation of compatible solutes (proline, soluble sugars), salt exclusion, structural changes, and regulation of membrane permeability (Hasegawa et al., 2000; Mansour and Salama, 2004; Munns and Tester, 2008; Chaves et al., 2009). Salt deteriorated soils can be rehabilitated using plant species and varieties that can tolerate high salt concentrations, and have always been a preferred choice for improving productivity in salt-affected soils. Some species belonging to the tropical *Casuarinaceae* family are widely used in reforestation programs. They are usually salt-tolerant pioneering trees that form root nodules in association with the nitrogen-fixing actinobacteria *Frankia*. Among the family, *Casuarina glauca* is seen as a promising species for preventing desertification, stabilizing coastal dunes, and restoring degraded soils in tropical and subtropical regions (Diem and Dommergues, 1990; Ribeiro et al., 2011; Sayed, 2011). Previous studies have reported that *C. glauca* tolerates up to 600 mM NaCl, a concentration above that of seawater (Batista-Santos et al., 2015; Scotti-Campos et al., 2016). The species is also an excellent raw material for the paper and pulp industries, being ideal for poles and scaffolding (Zhong et al., 2011). Because this species is very tolerant to high salinity and is considered a halophyte (Ribeiro-Barros et al., 2022), researchers are particularly interested in learning how it responds and adapts to salinity stress. In order to contribute to the understanding of *C. glauca* salt-tolerance mechanisms and its potential in rehabilitation programs, our study aimed at evaluating the effects of preconditioning to salinity stress on the physiological and biochemical traits of seedlings. Preconditioning is a simple and practical method that involves exposure in the nurseries to a given pretreatment, prior to outplanting in order to rapidly promote seedling traits associated with tolerance and adaptation (Villar-Salvador et al., 1999). The effectiveness of preconditioning lies in the fact that young plants are malleable and can usually thrive under stress conditions if they have undergone a prior period of stress (Villar-Salvador et al., 1999). However, this strategy has still not been studied in salt stress conditions, particularly in *C. glauca* plants and the potential of salt preconditioning to improve tolerance remains untapped. Therefore, we addressed if salt preconditioning is effective at promoting seedling traits associated with tolerance in *C. glauca* without impairing physiological vigor. To answer this question we (i) evaluated the impact of salinity on gas exchange variables, electrolyte leakage, chlorophyll fluorescence, and water relations parameters of preconditioned and non-preconditioned *C. glauca* plants, (ii) determined primary and secondary metabolic adjustments, and (iii) assessed gene expression profiles of some salt-related genes.

Material And Methods

Experimental conditions

Soil samples were taken from the saline fields of Kalaât Landalous, Tunisia (37° 3' 45" N, 10° 7' 6" E). The area has a semi-arid climate, with an annual rainfall of around 470 mm. The soil was collected from a depth of 30 cm, after removing the upper layer. The soil has a fine texture, silty clay to clay, a pH of 8.33, and electrical conductivity (EC) of 15 dS/m. A soil mix (30% sand, 70% potting soil) was used as the control soil.

The experiment was carried out at the National Institute of Research in Rural Engineering, Water and Forests (INRGREF) in Tunis (Tunisia) under greenhouse conditions (average daily temperature of 29°C, humidity 65%), during one year and using one-year-old *C. glauca* seedlings. A randomized complete block design with four blocks was laid out. Plants in each block were divided into three units, where each was assigned a treatment. In the first unit, *C. glauca* seedlings were cultivated in non-saline soil and used as control (C). In the second unit, seedlings were cultivated in the saline soil (NPrS). In the third unit, seedlings were subjected to a preconditioning treatment for 90 days (PrS) where they were first grown in washed river sand and irrigated every other day with Hoagland solution. Then, to avoid an osmotic shock, salt enhancement was gradually imposed through the addition of 50 mM NaCl per week to the nutrient solution until a concentration of 200 mM was reached. Finally, the seedlings were transferred to pots containing the saline soil. A total of 240 seedlings (4 blocks of 20 seedlings each x 3 treatments) were used in the experiment. The pots were filled with the same mass (7 kg) of saline or control soil (non-saline) and were irrigated to 75–85% of field capacity to prevent leaching of mineral elements.

Gas Exchange And Intrinsic Water Use Efficiency Measurements

Net photosynthesis (A), stomatal conductance (gs), transpiration (E), and intercellular CO₂ concentration (Ci) were measured after 2 h of light exposure (1800 μmol m⁻²s⁻¹) using a portable open-system infrared gas exchange analyzer based photosynthesis system (LI6400, LI-COR, Lincoln, NE, USA). Intrinsic water use efficiency (iWUE) was calculated as the ratio of photosynthetic assimilation rate (A) to stomatal conductance (gs) (Ehleringer et al., 1993)

Chlorophyll Fluorescence And Electrolyte Leakage Measurements

Minimal fluorescence (Fo) and maximal fluorescence (Fm), were evaluated in *C. glauca* needles after two hours of dark adaptation using a portable fluorometer (Plant Efficiency Analyzer, Hansatech, King's Lynn, Norfolk, UK). The potential quantum yield of photosystem II (PSII), which was expressed as Fv/Fm, was calculated using the following equation (Krause et al., 1991):

$$Fv / Fm = (Fm - F0) / Fm$$

Electrolyte leakage was measured following the method of Campos et al. (2003) using four randomly selected seedlings per treatment. Shoots were cut into 1 cm long fragments. These fragments were soaked

in sterile test tubes containing 20 mL of distilled water in the dark at 25°C for 24 h. Then, the free conductivity (LC) of the initial medium was measured using a conductivity meter (Cellox 325, Multiline P3 PH/LF-SET, WTW GmbH, Weilheim, Germany) and expressed in $\mu\text{S}\cdot\text{cm}^{-1}$. The test tubes were placed in a water bath at 100°C for 1 h to determine the maximum leakage of all electrolytes. After cooling, the total conductivity (TC) was measured. The electrolyte leakage (EL) was calculated using the following formulae:

$$\text{Electrolyte leakage}\%(EL) = [1 - (CL / CT)] \times 100$$

Water Relation Parameters

Water relation parameters were determined from pressure volume (P-V) curves using 4 randomly selected seedlings per treatment. Measurements were conducted using pressure chambers (PMS 1000, PMS Instrument Co., Corvallis, OR, USA) according to the method described by Ritchie (1984). Eleven pressure levels (starting at -0.2 MPa down to -5.2 MPa) were applied and each level was maintained for 10 min, as described by Abidine et al. (1993). Before taking the measurements, the twigs of each seedling were fully submerged in distilled water and allowed to reach full turgor in darkness at room temperature. The measurements were taken over 4 days. Within each day, 3 samples were analyzed. The pressure–volume curve permitted the estimation of a set of variables namely, the osmotic potential at saturation ($\Psi\pi^{100}$), the osmotic potential with loss of turgor ($\Psi\pi^0$), the relative water content at loss of turgor (RWC_0), the modulus of elasticity (ε_{max}) and the osmotic adjustment (OA). These variables ($\Psi\pi^{100}$, $\Psi\pi^0$, RWC_0 and AWC) were determined from the pressure–volume curves as described by Schulte and Hinckley (1985) and Abidine et al. (1993).

The ε_{max} was calculated as follows (Jones et al., 1980):

$$\varepsilon_{\text{max}} = (\Psi\pi^{100} - \Psi\pi^0) \times (1 - \text{AWC}) / (1 - \text{RWC}_0)$$

Osmotic adjustment (OA) was calculated as follows (Albouchi et al., 2016):

$$\text{OA} = \Psi\pi^{100}(\text{control}) - \Psi\pi^{100}(\text{treatment})$$

Chlorophyll, Proline, Soluble Sugars And Malondialdehyde (Mda) Content

Chlorophyll was determined using the method of Arnon (1949). One hundred mg of fresh shoots were ground in 10 ml of 80% acetone to evaluate the total chlorophyll concentration. The samples were incubated at 4°C overnight before being centrifuged at 10,000 g for 10 minutes. The extract was collected, and the supernatant absorbance was measured at 645 and 663 nm with a spectrophotometer (Shimadzu UV-1800 PC model Kyoto, Japan). The total chlorophyll content was calculated using Arnon (1949) equation and expressed as mg g^{-1} dry mass

$$\text{Chl} = 8.02 * \text{DO}(663) + 20.2 * \text{DO}(645) V / M$$

Where V is the volume of the total extract, M is the mass of the fresh material, and DO is the optical density (nm).

The proline concentration was evaluated according to the method described by Monneveux and Nemmar (1986). For that, 100 mg of dry powder of shoot samples were homogenized in 2 mL of 40% methanol and the mixture was heated in a water bath at 85°C for 60 min. One mL of the extract was taken after cooling and mixed with 1 mL of acetic acid, 25 mg of ninhydrin, and 1 mL of a combination containing 120 mL of distilled water, 30 mL of acetic acid, and 80 mL of orthophosphoric acid. The samples were then incubated in a water bath for 30 min, after the solution had cooled; 5 mL of toluene was added and followed by centrifugation at 12,000 g for 15 min. The supernatant was recovered and dehydrated by adding 10 mg of Na₂SO₄. A spectrophotometer was used to measure the optical density at 528 nm (Shimadzu UV-1800 PC model Kyoto, Japan). Proline concentrations were determined in $\mu\text{mol g}^{-1}$ dry matter.

To determine the concentration of soluble sugars, 100 mg of dry shoot samples were homogenized with 10 mL of 80% ethanol, as described by Albouchi et al., (1997). After 30 minutes in a 70°C water bath, the mixture was centrifuged for 15 minutes at 10,000 g. The anthrone-sulphuric acid method was used to determine the total soluble sugar concentration: 0.5 mL extract was mixed with 3 mL anthrone solution (200 mg anthrone in 100 mL concentrated sulphuric acid, W/W) and heated in a boiling water bath for 10 minutes. A spectrophotometer reading at 625 nm was used to estimate the sugar concentration. The results were calculated using a glucose standard and expressed as mg g^{-1} dry mass.

Malondialdehyde (MDA) content was measured using the method of Heath and Packer (1968) using 50 mg dry shoots homogenized in 2 mL of 1% Trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 10 min at 4°C. Then, 0.5 mL of the supernatant was mixed with 1.5 mL of thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 90°C for 20 min. After stopping the reaction on ice, the samples are centrifuged at 10,000 g for 5 min. The optical density was measured at 532 nm using a spectrophotometer. The MDA content was estimated by using an extinction coefficient of 155 /Mm/cm.

Phenolic Compounds

Total phenolic content was measured using Folin-Ciocalteu method described by Bursal and Gulçin (2011). A volume of 100 μL of each shoot and root extract was mixed with 1 mL of Folin-Ciocalteu reagent (diluted 10-fold) followed by the addition of 900 μL of 10% sodium carbonate. The mixture was kept for 90 min in the dark at room temperature. Later on, the absorbance was determined by spectrophotometry at 765 nm. The total phenolic content was expressed as gallic acid mg equivalents per gram of dry mass (mg GAE/g).

Flavonoid content was estimated according to the method of Dewanto et al. (2002). A volume of 250 μL of the extract was mixed with 75 μL of 5% NaNO₂, 150 μL of 10% AlCl₃ and 500 μL of 1 M NaOH with 5 min time interval between each solution and placed at room temperature for 15 min. The final volume was brought up to 2.5 mL with distilled water. The absorbance was measured by spectrophotometry at 510 nm (Shimadzu UV-1800 PC model Kyoto, Japan) using catechin as the standard. The results were expressed in mg catechin equivalent per gram of dry mass (mg CE/g).

Total condensed tannins were measured using the method described by Crich and Sun (1998). A volume of 500 µl extract was mixed with 3 mL of 4% vanillin and 1.5 mL of HCl. The mixture was incubated at room temperature for 15 minutes. The absorbance was measured by spectrophotometry at 500 nm (Shimadzu UV-1800 PC model Kyoto, Japan) using the tannic acid as the standard. The results are given in milligrams of tannic acid equivalents per gram of dry mass (mg TAE/g).

Genes Expression Of Salinity Tolerance-related Genes

Total RNA was isolated from branchlets using the innuPREP Plant RNA Kit (Analytik Jena, Germany) adding 1 µl β-mercaptoethanol to the lysis solution. Intactness of the extracted RNA was verified by electrophoresis on a 1.5% agarose gel by evaluating the integrity of the 28S and 18S ribosomal RNA bands and the absence of smears. To assure the absence of DNA contamination, all samples were also analyzed by standard PCR reactions using standard Ubiquitin primers (Table 1) under the conditions detailed in da Costa et al. (2015). cDNA was synthesized from 1 µg total RNA using the SensiFAST™ cDNA Synthesis kit (Meridian BioScience, USA), according to the manufacturer's recommendations. The presence of a single amplification product of the expected gene size was verified by electrophoresis on a 1.5% agarose gel. The expression of nine selected *C. glauca* genes [Adenine phosphoribosyltransferase (*CgApt*), Glyceraldehyde 3-phosphate dehydrogenase (*CgGAPDH*), Citrate Synthase (*CgCS*), Ascorbate peroxidase (*CgApx*), Ubiquitin (*CgUbi*), Glutamine synthetase (*CgGS*), Succinate dehydrogenase (*CgSHD*), copper zinc superoxide dismutase (*CgSOD1*), and glutathione peroxidase (*CgGPX1*)] was analyzed using clathrin adaptor complex subunit (*CgAp47*) as the reference gene (da Costa et al., 2015; Duro et al., 2016). All primer sequences are presented in Table 1. rt-PCR reactions were prepared using the SensiFAST™ SYBR No-ROX kit (Meridian BioScience, USA) according to the manufacturer's protocol. One negative control was included for each primer pair, in which cDNA was replaced by water. Reactions were carried out in 96-well plates using a qTOWER 2.2 Thermal Cycler (Analytik Jena, Germany) with the following parameters: hot start activation of the Taq DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s. A melting curve analysis was performed at the end of the PCR run by a continuous fluorescence measurement from 55 °C to 95 °C with sequential steps of 0.5 °C for 15 s (single peaks were obtained). Three technical replicates were used for each biological replicate.

Table 1
Primer sequences, characteristics and rt-PCR efficiencies of the selected genes

Gene Symbol	Gene name	Primer sequences (5'-3')	Accession number	Efficiency (%)
<i>CgApt</i>	Adenine phosphoribosyltransferase	F: CTGGGGAGGTTATTTTCGGAAG	FQ313257.1	100
		R: CAATCACAAGTGCACGCTCTC		
<i>CgGAPHD</i>	Glyceraldehyde 3-phosphate dehydrogenase	F: CACTTGAAGGGAGGTGCAAAG	FQ312704.1	89
		R: GTGCAGCTAGCATTGGAGATG		
<i>CgCS</i>	Citrate Synthase	F: TTTGGGCATCGTGTTTACAA	FQ327702	99
		R: AAAGTGCAGCCTTCTCCAAA		
<i>CgApx</i>	Ascorbate peroxidase	F: CCATTGATTGGTGTGAGGAA	FQ325996	92
		R: CTGGGAGAGACGCTTGAATC		
<i>CgUbi</i>	Ubiquitin	F: GCAGAGGTTGATTTTCGCTGG	FQ369009	100
		R: GTGGATTGCAGCCAATTCTTC		
<i>CgGS</i>	Glutamine synthetase	F: GACCTCACTCCGTACACCGA	FQ369458.1	99
		R: GATGTTCAACAGGCTTGGG		
<i>CgSHD</i>	Succinate dehydrogenase	F: CGAAGTTTGCAAAGCGTGTA	FQ323169	92
		R: GACGACACTGCAACTTCCAA		
<i>CgSOD1</i>	Copper zinc superoxie dismutase	F: GGAAGTGAGGGTGTCAAAGG	FQ364024	100
		R: TCCAGTTGACATGCAACCAT		
<i>CgGPX1</i>	Glutathione peroxidase	F: TCGAGGGTTTGAGATCTTGG	FQ320668	92
		R: AATTGGTGCTGCATTCTTCC		

Gene Symbol	Gene name	Primer sequences (5'-3')	Accession number	Efficiency (%)
<i>CgAp47*</i>	Clathrin adaptor complexes subunit	F: TCTTGAGGGTGAAATCGTTTCG	FQ318589.1	91
		R: TGATGCATAGCGCCTGTATAC		

Statistical analysis

Results in this study were averaged among four replicates with standard deviation (SD). For gene expression analysis, the relative expression ratio of a target gene was quantified based on its real-time PCR efficiencies and the crossing point (CP) difference of the unknown sample versus the control (0 mM NaCl) as described by da Costa et al. (2015). Significant differences between the means of different treatments were analyzed by one-way analysis of variance (ANOVA; $P < 0.05$) followed by the post-hoc Tukey test at a 5% significance level. All the data were analyzed by SPSS 22.0 software (IBM, Armonk, NY, USA).

Results

Net photosynthesis and gas exchanges

Salt stress significantly decreased the net photosynthesis in NPrS plants ($P = 0.0001$), with a reduction of 29 %, in relation to control (C) plants. Net photosynthesis in preconditioned plants (PrS) showed no differences in relation to the control (Fig. 1a; $P = 0.333$). Plants from the two treatments had significantly lower stomatal conductance (gs): 30 % for NPrS and 20 % for PrS compared to the control (Fig. 1b; $P = 0.0001$). Transpiration rate (E) exhibited no significant changes in the preconditioned plants ($P = 0.338$) but showed a significant decrease of 21 % in NPrS plants (Fig. 1c; $P = 0.003$). Similarly, the effect of salinity in the intercellular CO₂ concentration was significantly lower in NPrS plants ($P = 0.0001$) declining by 14 %, whereas it showed no significant differences in PrS plants in comparison with the control plants (Fig. 1d; $P = 0.092$).

Intrinsic water use efficiency, chlorophyll fluorescence and electrolyte leakage

Intrinsic water use efficiency (iWUE) showed no significant differences between treatments. (Fig. 2a; $P = 0.94$). However, the initial fluorescence (Fo) decreased with salinity, with an observed reduction of 7 % in preconditioned plants and 8 % in plants that were not preconditioned (Fig. 2b; $P = 0.0001$). Salinity did not significantly affect the quantum yield of PSII, as indicated by Fv/Fm, in either treatment (Fig. 2c; $P = 0.362$). In the saline soil, the membrane integrity remained unchanged in preconditioned plants (PrS) while in the absence of preconditioning, the Electrolyte leakage (EL) significantly increased (18 %) in relation to control plants (Fig. 2d; $P = 0.001$).

Water relations

The osmotic potential at full ($\Psi\pi_{100}$) and zero ($\Psi\pi_0$) turgor values were significantly more negative in stressed plants than in control plants (Fig. 3a, B; respectively $P= 0.001$ and $P= 0.001$). The decrease in $\Psi\pi_{100}$ and $\Psi\pi_0$ values was more pronounced in preconditioned plants (29 and 17 % respectively). Salinity also decreased the relative water content at loss of turgor (RWC₀; $P= 0.007$). The reduction was about 8% in NPrS and 21 % in PrS compared to the control (Fig. 3c). The apoplastic water content increased in stressed plants being 1.2-fold higher in PrS plants than in NPrS plants (Fig. 3d; $P= 0.006$). The modulus of elasticity significantly decreased by 199 % and 66 % for PrS and NPrS plants, respectively (Figure 3e; $P= 0.001$). Preconditioned plants showed an osmotic adjustment (OA) 1.6-fold higher than non-preconditioned ones (Fig. 3f; $P= 0.038$).

Effect of salinity on chlorophyll, proline, soluble sugars and malondialdehyde (MDA) content

The salt treatment resulted in a significant decrease in the concentration of total chlorophyll in both treatments (Fig. 4a; $P= 0.001$). NPrS and PrS plants had respectively 37 % and 18 % less chlorophyll than the control plants. The content in proline and soluble sugars also increased with salinity. The concentration of proline in PrS plants was significantly higher than in NPrS plants (Fig. 4b; $P= 0.002$). The content of soluble sugars increased from 63 mol g DM⁻¹ in controls to 74 and 78 mol g DM⁻¹ in NPrS and PrS plants, respectively (Fig. 4c $P= 0.0001$). On the contrary, the content of malondialdehyde (MDA) showed no significant differences between control and PrS plants, although it increased significantly in NPrS plants (39 %; Fig. 4d; $P= 0.0001$).

Total content of phenolic compounds

More total polyphenols were accumulated in NPrS plants than in the pre-conditioned plants (PrS), although the polyphenol content in both treatments increased in relation to the control (Fig. 5a; $P= 0.0001$), by 83 % in NPrS and 53 % in PrS in the shoots and by 73 % in NPrS and 23 % in PrS in the roots. The effect of salt on flavonoid content was similar to that on polyphenols in both treatments. As shown in Fig. 5b, the flavonoid content of NPrS plants increased significantly by 64 and 98 % in shoots ($P= 0.0001$) and roots ($P= 0.0001$), respectively. In pre-conditioned plants, the increase in shoots and roots was of only 28 and 10 %, respectively ($P= 0.0001$ and $P= 0.0001$). Tannin content increased under saline conditions as well, being significantly higher in NPrS plants (Fig. 5c, $P= 0.0001$ and $P= 0.0001$).

Genes expression of salinity tolerance-related genes

The nine candidate genes complied with the PCR requirements showing efficiencies ranging between 89 and 100 % (Table 1) The relative expression of Adenine phosphoribosyl transferase (*CgApt*), Citrate Synthase (*CgCS*), Ubiquitin (*CgUbi*), and Glutamine synthetase (*CgGS*) genes did not change significantly on the shoots of *C. glauca* from either of the two saline treatments in relation to control samples (Table 2). However, Glyceraldehyde 3-phosphate dehydrogenase (*CgGAPDH*), ascorbate peroxidase (*CgApx*), and glutathione peroxidase (*CgGPX1*) genes were significantly over-expressed in the PrS and NPrS plants in comparison to the control. Succinate dehydrogenase (*CgSHD*) and copper zinc superoxide dismutase (*CgSOD1*) were significantly over-expressed on the shoots of PrS plants, in relation to control samples (Table 2).

Table 2 Gene expression of Adenine phosphoribosyltransferase (CgApt), Glyceraldehyde 3-phosphate dehydrogenase (CgGAPHD), Citrate Synthase (CgCS), Ascorbate peroxidase (CgApx), Ubiquitin (CgUbi), Glutamine synthetase (CgGS), Succinate dehydrogenase (CgSHD), copper zinc superoxide dismutase (CgSOD1), and glutathione peroxidase (CgGPX1) on the shoots of preconditioned and non-preconditioned *Casuarina glauca* plants grown in saline soil

	<i>CgApt</i>	<i>CgGAPHD</i>	<i>CgCS</i>	<i>CgApx</i>	<i>CgUbi</i>	<i>CgGS</i>	<i>CgSHD</i>	<i>CgSOD1</i>	<i>CgGPX1</i>
Treatment 1	0,98	2.33*	0,64	4.53*	0,35	0,89	0,66	0,66	2.22*
Treatment 2	0,87	4.21*	0,61	6.44*	0,37	0,77	4.22*	4.72*	3.55*

* Indicates significant up-regulation $p < 0.05$

Discussion

In this work, we have analyzed the impact of long-term soil salinity stress on preconditioned and non-preconditioned *C. glauca* plants, through physiological and biochemical performance and the related expression of key target genes.

Gas exchange, intrinsic water use efficiency, chlorophyll fluorescence, and electrolyte leakage

Gas exchange and chlorophyll fluorescence are the major methods for plant photosynthetic study, particularly under environmental stress. Salinity has a significant impact on photosynthetic carbon metabolism via stomatal closure, which limits CO_2 diffusion to carboxylation sites, a decrease in mesophyll conductance to CO_2 , and metabolic limitations to leaf photochemistry (Loreto et al., 2003). According to the results presented in this study, salt stress significantly reduced stomatal conductance (g_s), net photosynthesis (A), transpiration (E), and intercellular CO_2 concentration (Ci) in NPrS plants. Batista-Santos et al. (2015) found that increasing NaCl levels in *C. glauca* resulted in a significant decrease in photosynthesis, stomatal conductance, and internal CO_2 concentration, associated with the concomitant reduction of the levels of key photosynthetic and respiratory enzymes (without inactivation). Preconditioning to salinity alleviated the stomatal restriction, enhanced transpiration and intercellular CO_2 concentration to a level similar to the plants grown under controlled conditions, and made the decrease in net photosynthesis less pronounced. Therefore, high intrinsic water use efficiency (iWUE) under salt stress indicates that the leaves (specifically, the chloroplasts) struggle to maintain a high photosynthetic performance despite significant stomatal closure. A similar result was observed Batista-Santos et al. (2015) which reported WUE levels $> 80\%$ in plants exposed to 200, 400, and 600 mM NaCl. According to Chaves et al. (2009), a decrease in stomatal conductance (g_s) may have stress-protective effects by allowing plant water conservation and improving plant iWUE. The decrease in gas exchange as a whole could be attributed to toxic Na^+ and Cl^- ions, which reduce photosynthetic electron transport (Ashraf et al., 2012). Photosynthesis inhibition by salinity seems to be partially associated with the PSII complex. Some studies

found that salt stress inhibited PSII activity (Netondo et al., 2004; Benzarti et al., 2012), while others found that PSII was highly resistant to salt stress (Lu et al., 2002; Chen et al., 2004; Tarchoune et al., 2012). Our results revealed no effect on the quantum yield of PSII and a slight decrease in the initial fluorescence (F_o) in either treatment which was consistent with the small net photosynthesis (A) decrease, particularly in PrS plants. Similar results were obtained by Batista-Santos et al. (2015) in *C. glauca* exposed to different NaCl levels. Also, Balti et al. (2021) showed that F_v/F_m was almost not affected by salt stress in eucalyptus. Overall, these findings point to a significant salt tolerance capacity of the *C. glauca* photosynthetic apparatus. Our results also showed that membrane integrity is maintained in PrS plants, further confirming high salinity tolerance (Mansour, 2013). While we noticed a significant increase in electrolyte leakage (EL) in NPrS plants, Bistgani et al. (2019) showed that EL and Na^+ levels in plants have a significant relationship. In fact, higher levels of Na^+ in plants result in increased lipid peroxidation and membrane damage (Banu et al., 2009). However, Scotti-Campos et al. (2016), found that *C. glauca* maintains membrane integrity at salt concentrations up to 400 mM NaCl. The preservation of membrane integrity could be linked to the accumulation of ions in the vacuole and/or the synthesis of compatible solutes in the cytosol, which prevented the loss of cellular turgor (Chaves et al., 2009; Ramalho et al., 2014).

Water relations

Salt-stressed plants exhibited significantly lower osmotic potential at full ($\Psi\pi^{100}$) and zero ($\Psi\pi^0$) turgor than the control plants. The values of $\Psi\pi^{100}$ and $\Psi\pi^0$ of PrS plants were significantly more negative than those of NPrS and control plants. The current findings are consistent with those of Batista-Santos et al. (2015), who showed that increasing NaCl levels decreased the osmotic potential corrected to full turgor in *C. glauca*. Navarro et al. (2007) suggested that the decrease in osmotic potential at full turgor may be due to the accumulation of Na^+ and Cl^- ions or can result from an accumulation of organic and/or inorganic solutes. According to Flowers et al. (2015), osmotic adjustment is possibly the main adaptive mechanism used by plants to limit the osmotic effects of salt stress. Also, Munns (2002) suggested that salt stress frequently causes osmotic adjustment, which is thought to be an important mechanism for maintaining water uptake and cell turgor under stress conditions. This is consistent with our findings showing a substantial increase in OA for PrS plants that was 1.6-fold higher than NPrS ones. A greater decrease in $\Psi\pi^{100}$ and $\Psi\pi^0$ for PrS plants, combined with greater osmotic adjustment, may grant them the ability to take up water, despite the increase in osmotic potential of the soil solution due to salinity (Patakas et al., 2002; Flowers et al., 2015). As a result, PrS can sustain their turgor for a long time before reaching the loss of turgor point (Abidine et al., 1993), which is compatible with reduced RWC_0 values and enhanced cell membrane flexibility. This allows gas exchange to be maintained for longer periods (Abidine et al., 1995), as seen in this study. Plants that live in environments with dynamic salinity changes may benefit from flexible cell walls (low ϵ) because cell walls stretch and contract to maintain osmotic equilibrium with the environment (Kirst 1990; Touchette 2014). The fact that PrS has a lower modulus of elasticity (ϵ) than NPrS further supports this concept. This flexibility allows the plant to experience significant variations in the water content of the apoplast without affecting the dynamic structure of the cell walls (Clifford et al., 1998). This is confirmed by the significant increase in the apoplastic water content (AWC) in preconditioned (PrS) plants, compared to the control and the non-preconditioned ones (NPrS).

Chlorophyll, proline, soluble sugars and malondialdehyde (MDA) content

The chlorophyll content was significantly reduced in non-preconditioned and preconditioned plants at least extent in these latter. This result was consistent with the findings of Claver et al. (2020) who found a considerable decrease in chlorophyll concentration in *C. equisetifolia* at various NaCl levels. The decrease in chlorophyll could be related to the accumulation of Na⁺ ions, which inhibits particular enzymes involved in the synthesis of photosynthetic pigments (Sayyad et al., 2016), and loss of chloroplast membranes (Ceccarelli et al., 2010), and damage of chloroplasts caused by oxidative stress. (Gill and Tuteja, 2010).

Plants synthesize a range of organic solutes, including proline, soluble sugars, and others, which are referred to as osmolytes. The accumulation of osmolytes in plants exposed to salt stress has been linked to the plants' ability to survive and adapt to salinity conditions (Slama et al., 2008). In fact, osmolytes are osmoprotectant solutes that improve the cell's ability to retain water without interfering with normal metabolism (Singh et al., 2015). Besides, they protect plants from oxidative damage by inhibiting ROS production (Saradhi and Mohanty, 1993). This is consistent with our findings showing a considerable accumulation of soluble sugar and proline under salt stress, with a higher accumulation in PrS plants. The results corroborate the findings of Jorge et al. (2019) showing the importance of the secondary metabolome, namely of the flavonoid-based antioxidant system, in complementing the scavenging of reactive oxygen species (ROS) associated with the ascorbate-glutathione cycle (Scotti-Campos et al., 2016; Jorge et al., 2017a,b, 2021). This highlights the importance of both enzymatic and non-enzymatic scavenging components in the control of oxidative stress and, thereby, their key role in stress tolerance enhancement. The high accumulation of proline confirms *C. glauca* salt tolerance and performance under saline conditions (Jorge et al., 2017a, Claver et al., 2020). It is worth noting that proline preferentially accumulates in the cytoplasm and is involved in the regulation of salt tolerance mechanisms such as cellular enzymes and structural protection and can contribute to osmotic adjustment (Sanchez et al., 2004). Under salt stress, MDA levels increased, although this effect was less noticeable in PrS plants. This may be due to its higher salt tolerance. These results agree with the findings of Scotti-Campos et al. (2016) which showed non-significant variations along the stress imposition in *C. glauca* plants. With different plant species, Shalata and Tal (1998), and Juan et al. (2005) have concluded that MDA increased considerably in salt-sensitive lines than in salt-tolerant lines. This increase in MDA incidence could be related to photosynthetic impairment, insufficient enzymatic antioxidant activity, and ascorbate declination (non-enzymatic antioxidant) (You et al., 2015; Nxele et al., 2017).

Phenolic compounds

Under salt stress, the number of phenolic compounds in the shoots and roots of both treatments increased. In NPrS plants, the increase was more noticeable. These findings suggest that salinity has a significant impact on the secondary metabolism of *C. glauca*, possibly as a defense mechanism and biochemical adaptation to environmental stress (Dixon and Paiva, 1995). Increasing phenolic compound content has been observed also in buckwheat sprouts under treatment with various concentrations of NaCl (Lim et al., 2012). Hajlaoui et al. (2009) suggested that NaCl stimulates the synthesis of new polyphenolic derivatives that are potent antioxidants. Phenolic contents are important protective components of plant cells (Ashraf

et al., 2010). It has also been reported by Parida et al. (2004) that high levels of phenolic compounds attenuate the ionic effects of NaCl. In previous investigations into numerous plants (e.g. Wahid and Ghazanfar, 2006; Ksouri et al., 2007), salt-resistant cultivars were shown to accumulate more polyphenols than salt-sensitive cultivars.

Effects of salinity and preconditioning on the levels of salt-tolerance gene transcripts

We analyzed the effect of both salt treatments on the expression of a set of genes coding for proteins that have been previously associated with response to salt stress (Duro et al., 2016; Fan et al., 2017; Graça et al., 2020). The expression of Glyceraldehyde 3-phosphate dehydrogenase (*CgGAPDH*), Ascorbate peroxidase (*CgApx*) and glutathione peroxidase (*CgGPX1*) increased significantly under salt stress, particularly in PrS. Plants produce significantly more ROS, including H₂O₂, in their chloroplasts and peroxisomes when exposed to salinity (del Rio et al., 2006). Ascorbate peroxidase catalyzes the conversion of H₂O₂ to H₂O and O₂ and scavenges ROS to protect plants from the toxic effects of ROS accumulation (Chen et al., 2015). The genes that code for APXs are therefore crucial for maintaining ascorbate (*AsA*) and glutathione (*GSH*) levels, which are directly or indirectly involved in maintaining high photosynthetic rates in plants under adverse environmental conditions (Foyer et al., 2011). According to Teixeira et al. (2006), the increase in APX mRNA levels in response to salinity stress may maintain the high activity of APX in the cytosol to protect cellular components from ROS-induced oxidative damage. Furthermore, Yin et al. (2019) showed that *PtomtAPX* is dual-targeted to both the chloroplast and mitochondria of *Populus tomentosa* and that it exhibits the same expression pattern under salt stress. In French bean seedlings, Nageshbabu et al. (2013) found that the expression of APX-coding genes was up-regulated by salinity and drought stresses, indicating their function in molecular regulation mechanisms. For example, anaerobic stress induces the expression of *GAPC3* and *GAPC4* in maize (Manjunath et al., 1997), while overexpression of the rice cytosolic gene *OsGAPC3* improves salt tolerance (Zhang et al., 2011). In response to salinity stress, numerous plant species have shown an increased accumulation of GPx transcripts probably to detoxify stress-induced ROS (Sreenivasulu et al., 2004; Islam et al., 2015). The accumulation of excess ROS within the cell is a common result of all the stresses that plants are subjected to (Ghosh et al., 2014). Our data showed that succinate dehydrogenase (*CgSHD*) and copper-zinc superoxide dismutase (*CgSOD1*) genes were overexpressed in PrS plants. According to Ruth et al. (2002), *SOD* genes are the first line of defense against oxidative stress. The protective effect of *SOD* genes against salt stress has been described by Tanaka et al. (1999) and Badawi et al. According to Tanaka et al. (1999), rice transformed with yeast *MnSOD* was tolerant to salt stress to around 100 mM NaCl. Badawi et al. (2004) transferred rice cytosolic Cu/ZnSOD to tobacco chloroplasts and the transformed tobacco plants could then withstand NaCl stress up to 300 mM. Shafi et al. (2015) demonstrated that *SOD* genes cause substantial lignin deposition in the vascular system and interfascicular cambium. The enhanced lignification, along with the accumulation of osmoprotectants (proline and soluble sugars), are also crucial mechanisms that boost salt stress tolerance. According to the genetic evidence provided by Gleason et al. (2011), *SDH* participates in the localization of mitochondrial ROS that regulates plant stress and defense responses. Also, Acevedo et al. (2013) demonstrated that drought up-regulates *IpSDH1* expression in a drought-tolerant *Ilex paraguariensis* genotype and that this up-regulation was associated with a significant increase in succinate dehydrogenase activity in the absence of

mitochondrial damage. Altogether the over-expression of these antioxidant genes would explain the maintenance of a high photosynthetic rate and the chlorophyll content in *C. glauca* plants under saline stress, in particular in preconditioned plants. The results are supported by the proteomic analysis in *C. glauca* showing a remarkable pattern of accumulation of proteins involved in photosynthetic metabolism, and oxidative stress response, associated to two interacting networks: metabolic pathways and biosynthesis of secondary metabolites; and protein processing and export, carbon metabolism, and peroxisomal metabolism (Graça et al., 2020; Ribeiro-Barros et al., 2022). The overall set of results suggest that under salinity stress conditions, the triggered protective mechanisms might prevent a significant impact at the photochemical efficiency and the biochemical performance levels in *C. glauca*, explaining its high performance in saline soils.

Conclusion

This study showed that salinity induced physiological and biochemical changes in *C. glauca* plants, likely representing an adaptive response to salt stress. Its tolerance could be attributed to the plant's ability to maintain membrane stability and relevant metabolic functions such as photosynthesis, stomatal conductance, and chlorophyll fluorescence; balanced osmolyte content, such as proline and soluble sugars; increased secondary metabolism as shown by the enhancement of phenolic content, and increased expression of salt tolerance related genes such as *GPX1*, *SOD1*, *SHD*, *APX*, and *GAPHD*. The results of this experiment also demonstrated that *C. glauca* can acquire a higher tolerance to salt stress after receiving preconditioning treatments. Our results suggest that the use of preconditioning can be a promising practice for improving the physiological and biochemical quality of seedlings produced in forest nurseries, their performance, and their tolerance to salt stress. Our results also demonstrate the importance of conducting physiological and molecular analysis simultaneously to provide a better understanding of the response of plants to abiotic stress. In general, *C. glauca* was shown to be appropriate for application on salinity-affected soils. However, because our research was conducted in a controlled context, it is necessary to repeat the experiments in a natural (uncontrolled) environment to confirm our findings.

Declarations

Author Contribution statement:

Ines Laamari: Methodology, Formal analysis, Writing - Original Draft.

Isabel Marques: Writing - Review & Editing, Formal analysis.

Ana I. Ribeiro-Barros: Writing - Review & Editing, Visualization.

Zoubeir Béjaoui: Conceptualization, Project administration.

Mejda Abassi: Validation, Supervision, Project administration.

All authors reviewed the manuscript.

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Figures

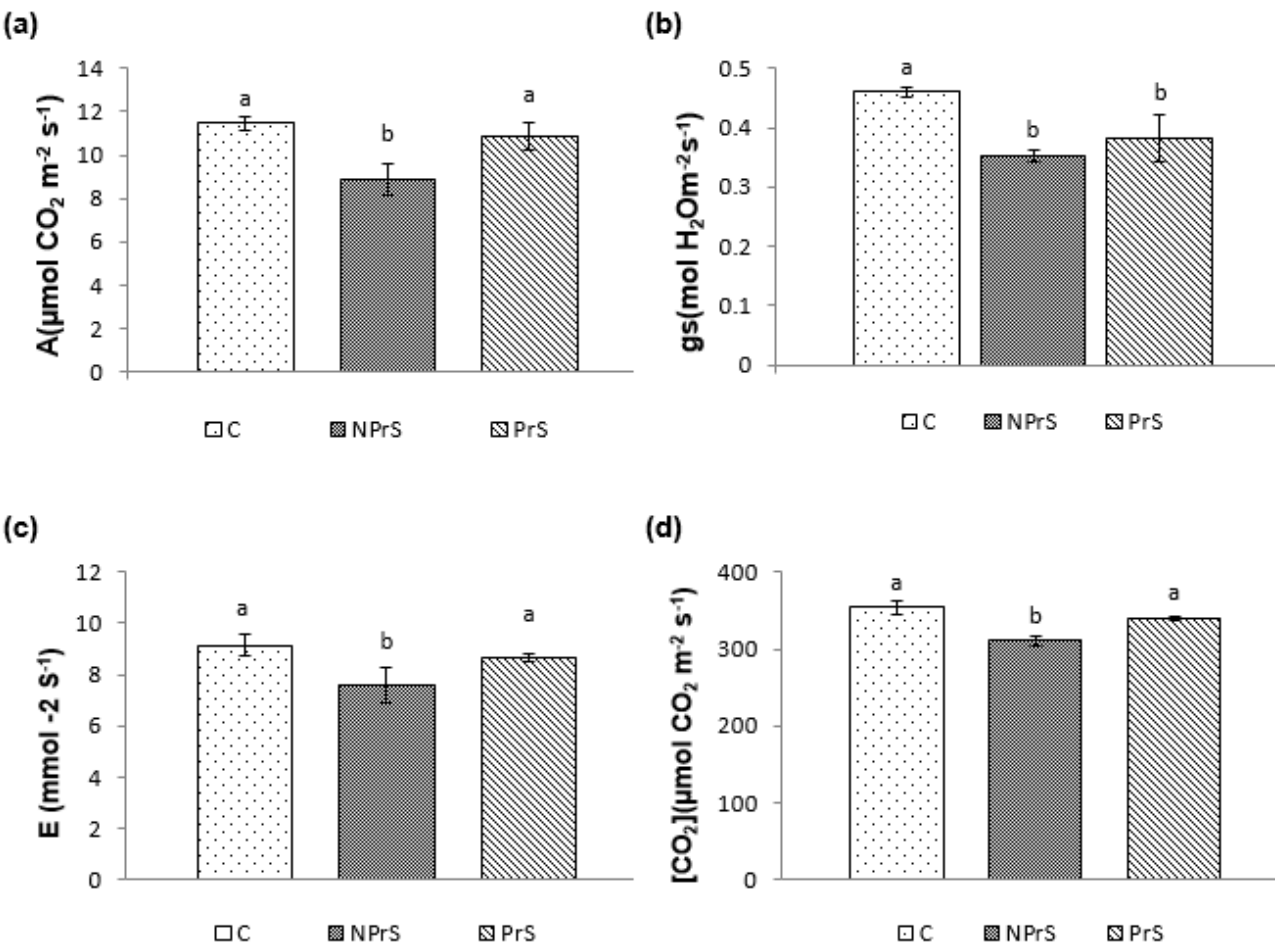


Figure 1

(a) Net photosynthesis (A), (b) stomatal conductance (g_s), (c) transpiration (E) and (d) intercellular CO_2 concentration (C_i), of preconditioned and non-preconditioned *Casuarina glauca* plants grown in Saline and control soil. Means ($\pm$ SD; $n = 4$) with different letters indicate significant differences between treatments according to Tukey's tests at $P \leq 0.05$

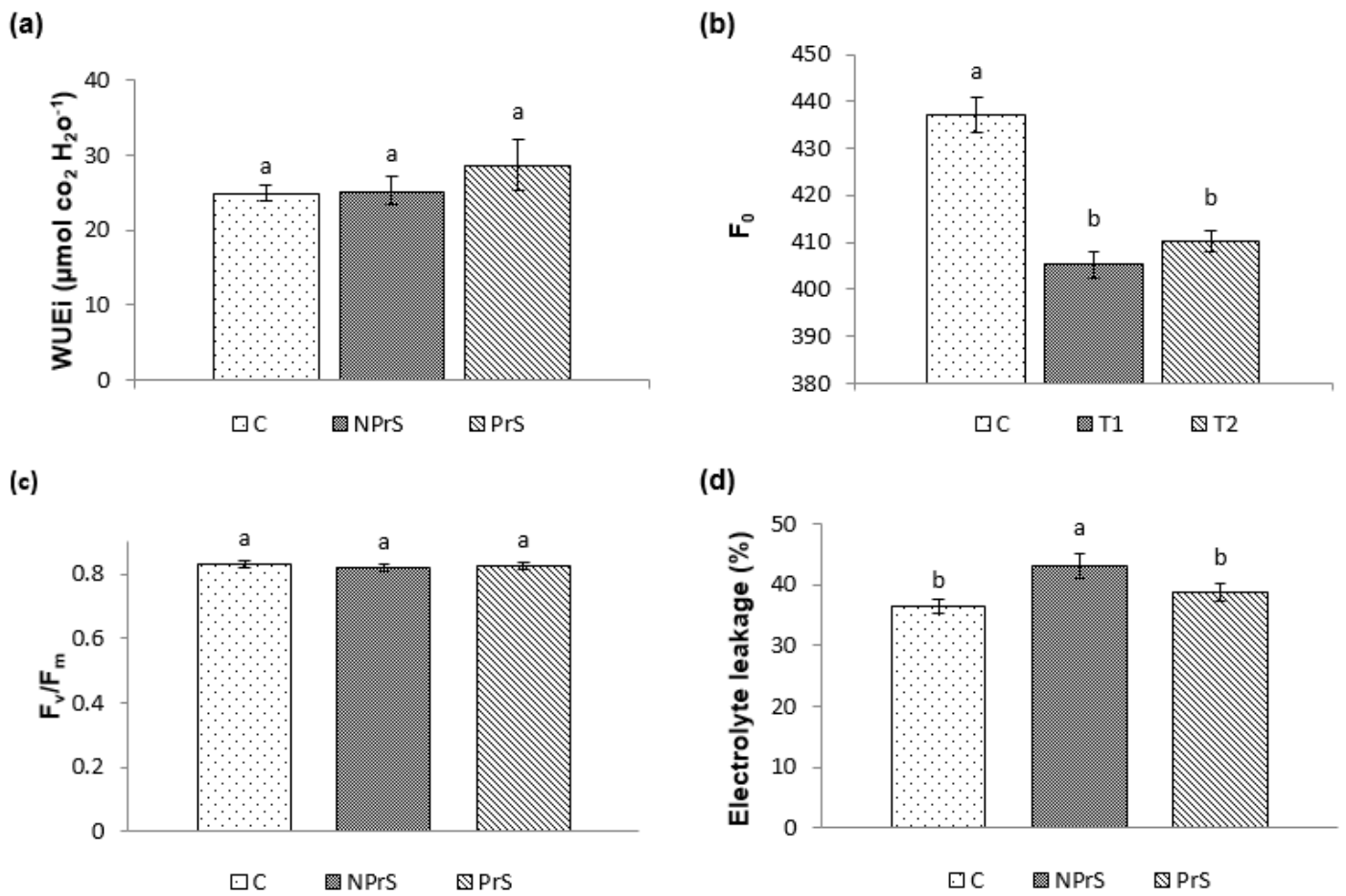


Figure 2

(a) Intrinsic Water-Use Efficiency (WUEi), (b) Initial fluorescence (F_0), (c) Maximum quantum yield of PSII (F_v/F_m) and (d) Electrolyte leakage of preconditioned and non-preconditioned *Casuarina glauca* plants grown in Saline and control soil. Means ($\pm$ SD; $n = 4$) with different letters indicate significant differences between treatments according to Tukey's tests at $P \leq 0.05$

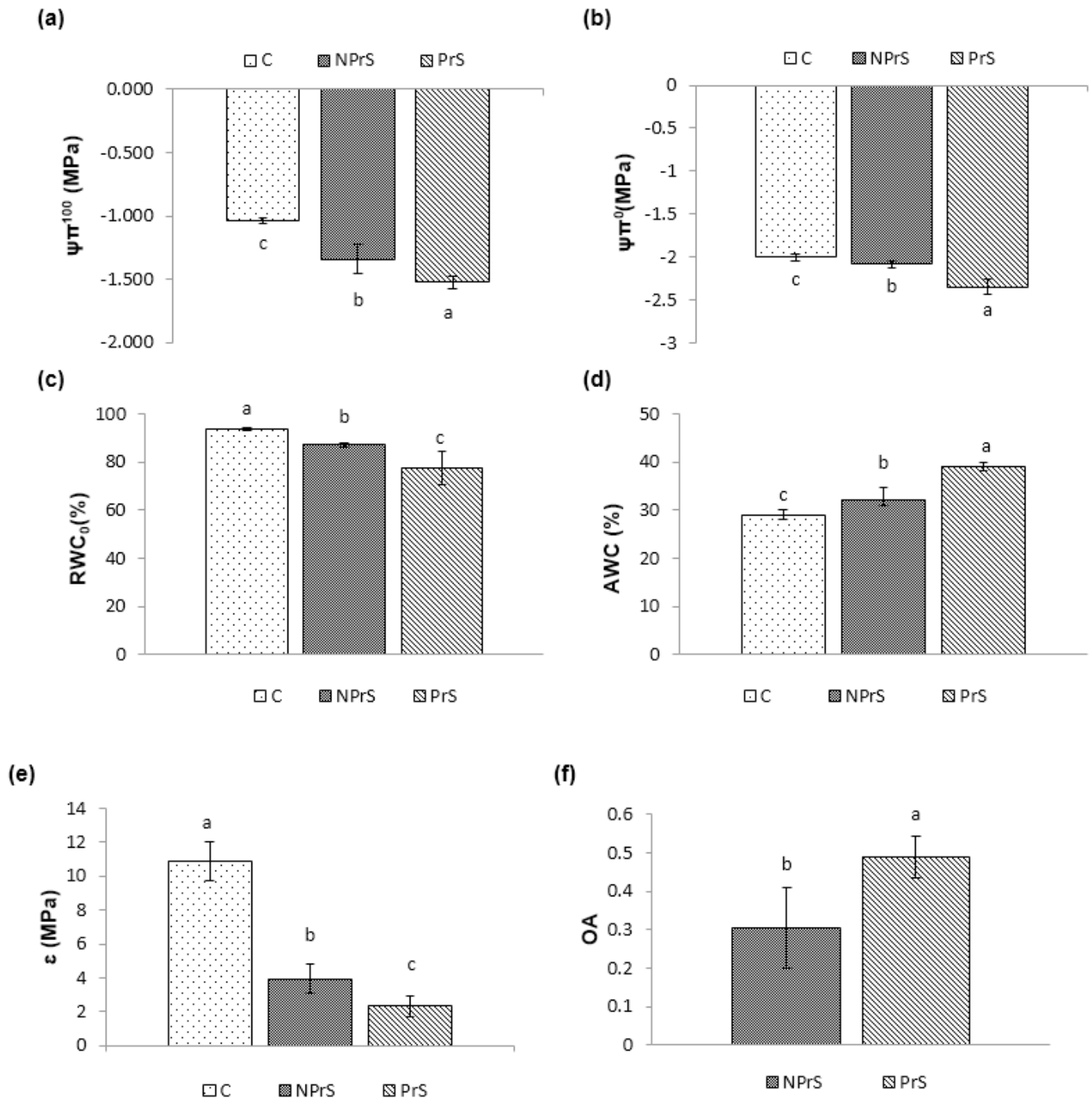


Figure 3

(a) Osmotic potential at saturation ($\Psi\pi^{100}$), (b) osmotic potential with loss of turgor ($\Psi\pi^0$), (c) relative water content at loss of turgor (RWC_0), (d) apoplastic water content (AWC), (e) modulus of elasticity (ϵ_{max}) and (f) the osmotic adjustment (OA) of preconditioned and non-preconditioned *Casuarina glauca* plants grown in Saline and control soil. Means ($\pm$ SD; $n = 4$) with different letters indicate significant differences between treatments according to Tukey's tests at $P \leq 0.05$

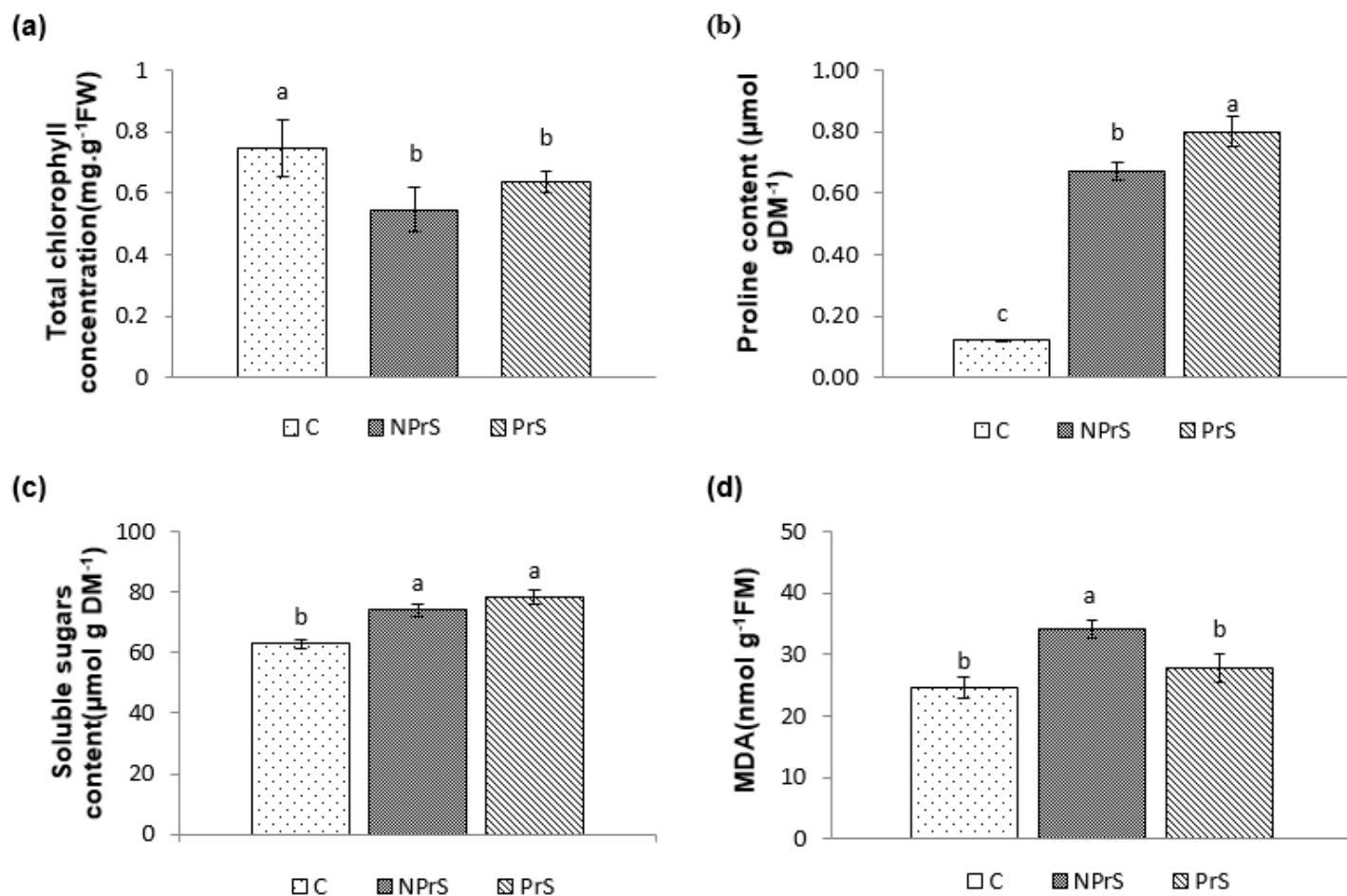
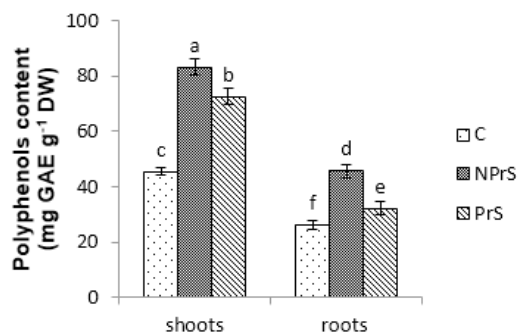


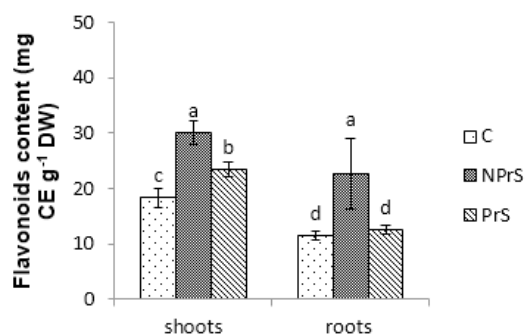
Figure 4

(a) Chlorophyll, (b) Malondialdehyde (MDA), (c) Proline and (d) soluble sugars content of preconditioned and non-preconditioned *Casuarina glauca* plants grown in saline and control soil. Means ($\pm$ SD; $n = 4$) with different letters indicate significant differences between treatments according to Tukey's tests at $P \leq 0.05$

(a)



(b)



(c)

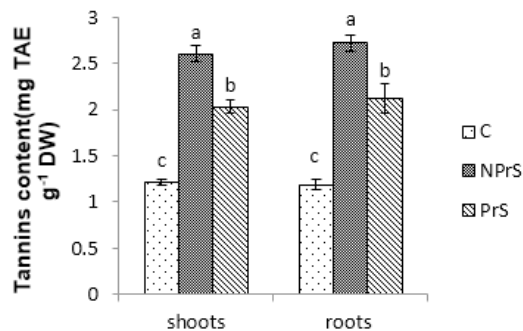


Figure 5

(a) Total contents of polyphenol, (b) flavonoids and (c) tannins in shoots and roots of preconditioned and non-preconditioned *Casuarina glauca* plants grown in saline and control soil. Means ($\pm$ SD; n = 4) with different letters indicate significant differences between treatments according to Tukey's tests at $P \leq 0.05$